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Increased monocyte adhesion by endothelial expression of VCAM-1 missense variation *in vitro*

Boris Schmitz [a,b], Peter Vischer [c], Eva Brand [b], Klaus Schmidt-Petersen [d], Adelheid Korb-Pap [e], Katrin Guske [b], Johanna Nedele [b], Michael Schelleckes [b], Jan Hillen [e], Alois Rötrige [a], Thomas Simmet [d], Martin Paul [f], François Cambien [g], Stefan-Martin Brand [a,*]

[a] University Hospital Münster, Institute of Sports Medicine, Molecular Genetics of Cardiovascular Disease, Horstmarer Landweg 39, D-48149 Münster, Germany
[b] University Hospital Münster, Internal Medicine D, Nephrology, Hypertension and Rheumatology, Münster, Germany
[c] Leibniz-Institute for Arteriosclerosis Research at the University of Münster, Germany
[d] Institute of Pharmacology of Natural Products and Clinical Pharmacology, Ulm University, Ulm, Germany
[e] Institute of Experimental Musculoskeletal Medicine, University Hospital Münster, Germany
[f] Maastricht University, Faculty of Health, Medicine, and Life Science, Maastricht, The Netherlands
[g] INSERM, UMR S937, Université Pierre et Marie Curie, Paris, France

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ABSTRACT

Objective: In whole genome and single gene analyses, genetic variation at the *vascular cell adhesion molecule-1* (*VCAM-1*) locus has been associated with inflammatory disease and stroke in sickle cell anaemia. In the current work, we investigated the functional impact of *VCAM-1* missense variants and their effect on cell–cell adhesion.
Methods and results: To determine the functional *in vitro* relevance of five missense *VCAM-1* variants (S318F; T384A; G413A; L555V; I716L), we generated wild type and single variant VCAM-1 forms [318F, 384A, 413A, 555V, 716L] in EA.hy926 endothelial cells. Real-time PCR, western blot and ELISA analyses revealed significant differences in mRNA and protein levels for *VCAM-1* variants. Monocytic cell lines THP-1 and U937 showed significantly increased adhesion to endothelial cells overexpressing VCAM-1 forms 318F, 555V and 716L compared to those overexpressing wild type VCAM-1 ($p < 0.05$).
Conclusions: VCAM-1-dependent cell adhesion to endothelial cells *in vitro* is significantly increased when expressing *VCAM-1* missense mutations 318F, 555V and 716L. The underlying mechanism involves altered VCAM-1 protein levels and function. This observation may be of particular relevance for chronic inflammatory pathophysiologic conditions involving cell–cell adhesion such as atherosclerosis and other proinflammatory conditions.

1. Introduction

Vascular cell adhesion molecule-1 (VCAM-1, CD 106) is a glycosylated cell surface protein expressed on different cell types, including endothelial cells and fibroblasts. VCAM-1 is a member of the immunoglobulin (Ig) gene superfamily with seven cell surface Ig-like domains, a hydrophobic transmembrane domain and a short cytoplasmic domain of 19 amino acids [1]. Human *VCAM-1* is a single copy gene at chromosome 1p32-p31, and contains nine exons spanning ~22.8 kb [2]. As a result of alternative splicing of exon 5, two different VCAM-1 transcripts have been described [3]. Elevated expression of VCAM-1 on endothelial cells depends on the presence of cytokines and mediates leukocyte accumulation in inflamed tissues [4]. The predominant receptor for VCAM-1 is the integrin α4β1 (very late activation antigen 4 [VLA-4]), while α4β7 and *Plasmodium falciparum*-infected erythrocytes also function as VCAM-1 receptors [5–7]. Animal knock-out studies indicated a major role of VCAM-1, compared to ICAM-1 or the selectins, in the

* Corresponding author. Tel.: +49 251 83 52996; fax: +49 251 83 35387.
E-mail addresses: stefan-martin.brand@ukmuenster.de, s.m.brand@uni-muenster.de (S.-M. Brand).

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pathogenesis of early atherosclerosis [8,9]. As genetic variation at the *VCAM-1* locus has been associated with stroke in patients suffering sickle cell disease [10,11], we hypothesized that missense variants of the *VCAM-1* gene may influence VCAM-1 mRNA/protein expression or protein function. To verify the presence of genetic variants in the coding region of *VCAM-1* in a European population, we scanned 190 chromosomes from Caucasian individuals of the Etude Cas-Temoins sur l'Infarctus du Myocarde (ECTIM) study. Subsequent *in vitro* expression studies included real-time PCR, western blot, ELISA and subsequent cell–cell adhesion assays.

2. Methods

2.1. VCAM-1 missense mutations

Genomic DNA from 95 Caucasian individuals (190 alleles) of the ECTIM Study (originally described in 1992 [12]) was prepared from white blood cells to verify the presence of *VCAM-1* missense variants in a European population. Informed written consent was obtained from all study subjects and the study was approved by the respective local ethic committees. This sample size yielded a greater than 95% detection rate for genetic variants with a minor allele frequency of 1% [13]. Overlapping *VCAM-1* fragments were amplified to cover 2318 bp, representing the entire coding region and exon/intron boundaries. Amplifications were performed using 20 ng of DNA, 25 pmol of each primer and 0.2 U *Taq* polymerase (GoTaq DNA-Polymerase; Promega, Mannheim, Germany). Specific amplification protocols for each primer pair can be obtained at our web site (http://genecanvas.ecgene.net). Single-Strand Conformation Polymorphism (SSCP) analysis was performed as previously described [14]. DNA presenting different migration patterns on the polyacrylamide gels were subsequently sequenced twice using an automated sequencing device (ABI Prism 377, Perkin Elmer, USA).

2.2. Cell culture

The human vascular endothelial cell line EA.hy926 [15] was maintained in DMEM (Sigma–Aldrich, Munich, Germany) with 10% conditioned fetal calf serum (FCS; PAA, Cölbe, Germany), penicillin (100 units/ml), streptomycin (100 ng/ml), and L-Glutamine (2 mmol/ml, all Sigma–Aldrich). The human monocytic cell lines THP-1 and U937 were cultured in RPMI 1640 (Sigma–Aldrich) with 10% FCS, penicillin (100 units/ml), streptomycin (100 ng/ml) and L-Glutamine (2 mmol/ml). THP-1 cell medium was supplemented with MEM amino acid mix (1%, Sigma–Aldrich) and sodium pyruvate (1%, Sigma–Aldrich). Different transfection reagents were used for EA.hy926 cells (jetPEI, Polyplus-Transfection, PeqLab, Erlangen, Germany; Nanofectin, PAA) according to manufacturers' instructions. Cells were stimulated with TNF-α (20 ng/ml, 4 h; Sigma–Aldrich).

2.3. Generation of mutant VCAM-1 cDNA constructs

The *VCAM-1* coding sequence (Acc#: NM_001078), including the full-length 3′-UTR, was subcloned into the eukaryotic expression vector pcDNA3.1 (Invitrogen, Karlsruhe, Germany). Mutations were introduced using the Quick-Change site-directed mutagenesis kit (Stratagene, La Jolla, USA), generating the non-synonymous variants 318F, 384A, 413A, 555V and 716L. All vectors were sequenced to ensure sequence accuracy and identity.

2.4. Isolation of total RNA, cDNA generation and PCR

Total RNA was extracted 24 h after transfection of cells with *VCAM-1* constructs using the RNeasy kit (Qiagen, Hilden, Germany) and up to 5 μg of total RNA was applied as template for cDNA synthesis by SuperScript II (Invitrogen, Karlsruhe, Germany). *VCAM-1* quantitative real-time PCR was performed using Power SYBR Green (Applied Biosystems) and primer 5′-TTCCCTAGA-GATCCAGAAATCGAG-3′ (sense) and 5′-CTTGCAGCTTACAGTGACA-GAGC-3′ (antisense; [16]), on an Applied Biosystems 7500 Fast Real-Time PCR system. Samples were run in duplicates under standard real-time PCR conditions. Relative quantification was calculated using the ΔΔCt method. GAPDH (sense primer 5′-CTGCACCACCAACTGCTTAGCAC-3′; antisense primer 5′-GTGATGG-CATGGACTGTGGTCATGAG-3′) was used as endogenous control. The absence of non-specific amplification was confirmed by agarose gel electrophoresis of PCR amplicons and generation of melting curves using the Applied Biosystems real-time PCR system software.

2.5. Western blot analysis

Whole cell lysates were prepared 24 h after transfection with *VCAM-1* constructs using RIPA buffer containing "Complete" protease inhibitors (Roche, Mannheim, Germany). After separation by SDS-PAGE, membranes were detected with human VCAM-1 monoclonal antibody (1:1000; Santa Cruz, Sta. Cruz, USA, sc-13160) and anti-mouse secondary antibody (1:2000; GE Healthcare). The open source software ImageJ (http://rsbweb.nih.gov/ij/) was used for band intensity quantification.

2.6. VCAM-1 ELISA

Overall cellular VCAM-1 protein concentrations (RIPA extracts) as well as extracellular soluble VCAM-1 levels from three independent experiments were determined by ELISA (Uscn Life Science, Houston, USA, E90547Hu) 24 h after transfection of EA.hy926 cells with *VCAM-1* expression vectors. Complete cell culture supernatants (2 ml) were collected, spun down and 100 μl were applied for ELISA. Twenty μg of total cellular protein were analysed. Horseradish Peroxidase reaction was quantified at 450 nm following manufacturers' instructions on a 96-well plate reader (Infinite 200 PRO NanoQuant, Tecan, Männedorf, Switzerland).

2.7. Cell–cell adhesion assays

Cell–cell adhesion experiments were conducted using EA.hy926 cells and THP-1 or U937 monocytes under basic conditions and after stimulation with TNF-α. EA.hy926 cells were transiently transfected with either *VCAM-1* wild type or mutant constructs in triplicates and adhesion experiments were performed after 24 h. THP-1 and U937 cells were fluorescence-labelled using Calcein-AM (100 μM, 1×10^6 cells; R&D Systems). Endothelial cells and monocytes were co-incubated for 30 min at 37 °C. Non-adherent cells were then removed, wells were washed twice with PBS and cells were lysed subsequently. Spectrofluorometric quantification was performed at 492 nm (excitation) and 535 nm (emission) on a 96-well plate reader (Infinite 200 PRO NanoQuant, Tecan).

2.8. Statistical analysis

GraphPad Prism Student's *t*-test and one-way ANOVA was used for statistical analysis with significance set to $p < 0.05$.

3. Results

3.1. Screening for VCAM-1 variants

Screening of 95 Caucasian individuals of the ECTIM study confirmed the presence of five *VCAM-1* coding variants (Fig. 1), two

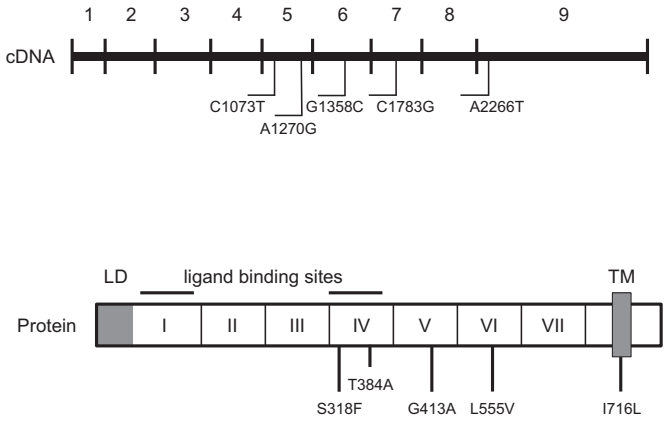

Fig. 1. Schematic representation of VCAM-1. Genetic variants and exons are indicated in the upper panel, corresponding amino acid changes in the lower panel. VCAM-1 contains a leader peptide (LD, grey), seven extracellular immunoglobulin-like domains (I–VII), a transmembrane region (TM, grey) and a C-terminal tail of 18 amino acids. Integrin binding sites are located in domains I and IV.

of which were located in exon 5 (S318F [rs3783611], T384A [rs3783612]), one in exon 6 (G413A [rs3783613]), one in exon 7 (L555V [rs114207303]) and one in exon 9 (I716L [rs3783615]). For subsequent molecular biological analyses, minor alleles were introduced separately into full-length *VCAM-1* cDNA including the 3′-UTR, generating VCAM-1 318F, 384A, 413A, 555V and 716L.

3.2. Mutant VCAM-1 transcript levels

Initially, we used the VCAM-1-negative cell line CV1 to determine potential differences in VCAM-1 mRNA expression after introduction of the identified genetic variants, following the hypothesis that missense variants may affect transcription efficiency, mRNA processing and transcript stability. Using PCR amplification and densitometric quantification, we did not detect any significant differences in VCAM-1 transcript levels in CV1 cells (Supplemental Fig. 2). We performed additional experiments in the vascular endothelial cell line EA.hy926, which is capable of expressing VCAM-1. In addition, EA.hy926 cells exhibit functional anti-inflammatory pathways such as peroxisome proliferator-activated receptors [17] and miRNA-126 [18], which might influence the expression of *VCAM-1* mutants. Using real-time PCR analysis, we detected significant differences in VCAM-1 transcript levels with respect to the overexpressed variant in EA.hy926 cells. With the exception of 716L, all mutant constructs were associated with significantly elevated VCAM-1 expression levels above wild type ($p < 0.01$; Fig. 2). While the VCAM-1 mRNA levels of cells expressing 413A was elevated up to 2-fold, VCAM-1 mRNA levels of cells expressing 318F, 384A and 555V were elevated up to 4-fold. Under inflammatory TNF-α conditions, the VCAM-1 mRNA levels of cells expressing 318F were significantly increased up to 6-fold compared to wild type ($p > 0.05$), while cells expressing 384A did show a trend towards strong VCAM-1 mRNA level increase (Fig. 2). The VCAM-1 mRNA levels of cells expressing 555V and 716L were also significantly increased under TNF-α conditions as seen at basic conditions.

3.3. Mutant VCAM-1 protein levels

To determine potential differences in VCAM-1 protein isoform levels and VCAM-1 glycosylation, which may be affected by the identified *VCAM-1* missense variants, western blot analyses was used. We detected three prominent VCAM-1 protein bands of ~50 kDa, ~75 kDa and ~100 kDa (Fig. 3). Densitometric analysis of VCAM-1 protein in relation to β-actin bands did not reveal any significant differences upon overexpression of individual VCAM-1 variants. Using the more sensitive ELISA for the determination of total cellular VCAM-1, we observed that VCAM-1 protein levels of cells overexpressing 716L was significantly increased by 50% compared to wild type ($p < 0.01$), while cellular VCAM-1 levels for cells expressing all other variants remained constant (Fig. 4A). The significant increase of VCAM-1 in cells overexpressing 716L was also observed under TNF-α conditions ($p < 0.01$; Fig. 4B). To account for a potential effect on soluble VCAM-1, conditioned media of transfected cells were also analysed by ELISA, but no significant difference was detected for any VCAM-1 variant (Fig. 4C/D).

3.4. Cell–cell adhesion assays

Any of the here observed differences in VCAM-1 mRNA and protein expression may alter VCAM-1 exposure at the cellular surface. Moreover, amino acid changes in functionally important VCAM-1 protein domains such as ligand binding domain 4, harbouring variants S318F and T384A or the transmembrane domain I716L, may influence monocyte adhesion to endothelial cells. To address this functional aspect, we performed cell–cell adhesion assays, using two different monocytic cell lines, THP-1 and U937, and endothelial cells transiently transfected with *VCAM-1* missense variants. This approach revealed that THP-1 adhesion was significantly enhanced in the presence of 318F, 555V and 716L compared

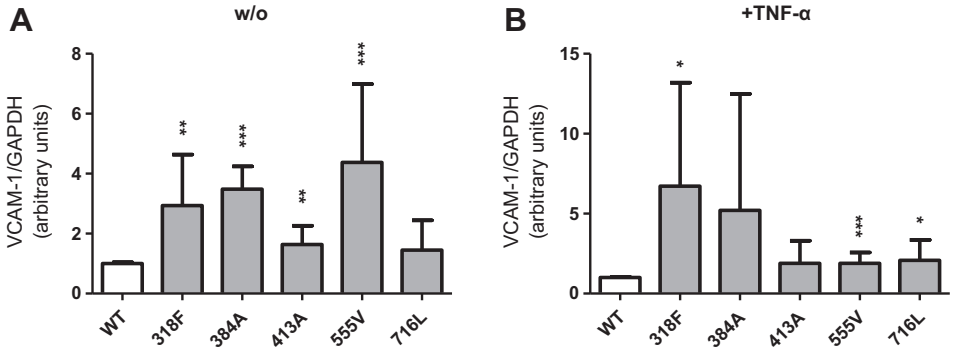

Fig. 2. *VCAM-1* variants significantly increase VCAM-1 mRNA expression in endothelial cells. (A) VCAM-1 mRNA levels of endothelial cells overexpressing transcripts 1073T (318F), 1270G (384A), 1358C (413A) and 1783G (555V) were significantly increased ($p < 0.01$) compared to wild type (wt) under basic conditions (w/o). (B) TNF-α stimulation resulted in a significant ($p < 0.05$) increase of VCAM-1 mRNA levels of endothelial cells overexpressing transcripts 1073T (318F), 1783G (555V) and 2266T (716L). *VCAM-1* expression was determined via real-time PCR 24 h after transfection ($n = 3$) and normalized to the endogenous control GAPDH. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$.

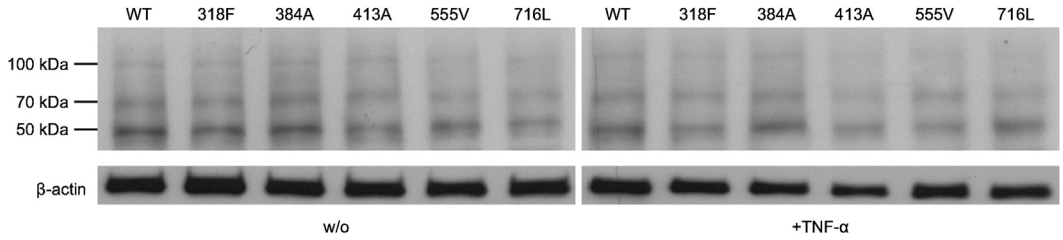

Fig. 3. Cellular VCAM-1 protein levels were analysed by western blot. No significant difference was observed for VCAM-1 protein levels of endothelial cells overexpressing VCAM-1 variants in western blot analysis. The three detected VCAM-1 protein bands of ~50, ~70 and 100 kDa remained constant under basic (w/o) and TNF-α conditions for all analysed mutant VCAM-1 forms compared to wild type. VCAM-1 protein was determined via western blot 24 h after transfection. β-actin served as loading control. Western blot is representative for experiments ($n = 3$).

to wild type ($p < 0.01$), yielding up to 60% more adherent monocytes with respect to endothelial cells overexpressing 555V (Fig. 5A). This effect was also observed under TNF-α conditions except for 716L (Fig. 5B). Endothelial overexpression of 318F and 716L also affected adhesion of U937 cells significantly ($p < 0.05$, compared to wild type) while no effect of 555V was observed (Fig. 5C). No significant differences in U937 cell adhesion were observed for TNF-α-treated endothelial cells (Fig. 5D). Interestingly, enhanced extracellular VCAM-1 protein levels were also observed in immunofluorescence microscopy of endothelial cells expressing 318F and 555V when compared to wild type (Supplemental Fig. 1).

4. Discussion

In the present study, we investigated the molecular biologic functionality of five *VCAM-1* missense variants *in vitro*. We observed considerable effects on VCAM-1 mRNA and protein expression and detected significantly increased adhesion of two human monocytic cell lines to endothelial cells in the presence of VCAM-1 forms 318F, 555V and 716L.

In contrast to other cell adhesion molecules including ICAM-1 and the selectins, VCAM-1 has been shown to be critical for early atherosclerotic lesion development [8,9]. The underlying mechanism involves monocyte adhesion to the endothelial lining of the vessel wall with subsequent infiltration of the vessel, differentiation into macrophages, oxLDL uptake and transformation into lipid-laden foam cells [19]. *VCAM-1* missense mutations may potentially affect this process through alteration of protein levels or protein function. With respect to the underlying mechanisms, *VCAM-1* variants inserting in known functional domains of the protein are more likely candidates for *in vitro* effects on cell adhesion. VCAM-1 contains two Ig-like modules comprising the Ile-Asp-Ser-Pro-Leu (IDPSPL) recognition sequence interacting with α4β1 [20]. The first Ig-like module (domain I) contains the core integrin-recognition sequence, while a

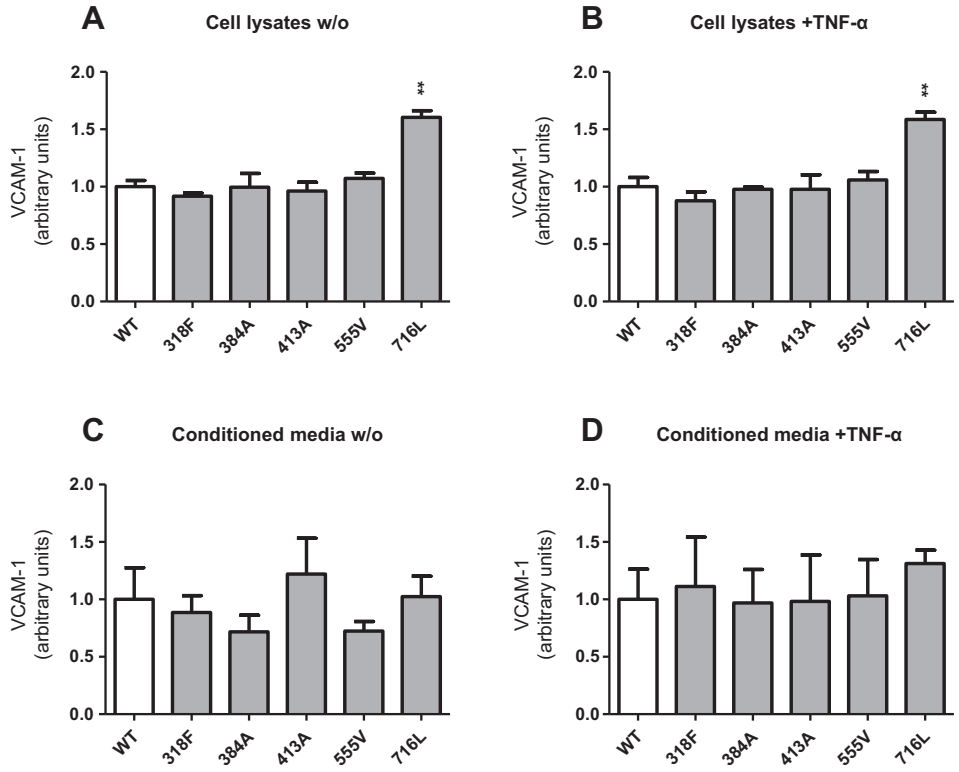

Fig. 4. VCAM-1 protein levels were determined by ELISA. (A) Total cellular VCAM-1 levels were significantly ($p < 0.01$) increased upon overexpression of variant 716L compared to wild type (wt) under basic conditions (w/o). (B) Comparable results were observed under TNF-α conditions ($p < 0.01$, 716L compared to wt). (C/D) No significant differences for soluble VCAM-1 levels in conditioned media were observed for any of the present VCAM-1 variant. ELISA was performed 24 h after transfection of endothelial cells. Results are shown as standard deviation of the mean of three independent experiments. $^{**}p < 0.01$.

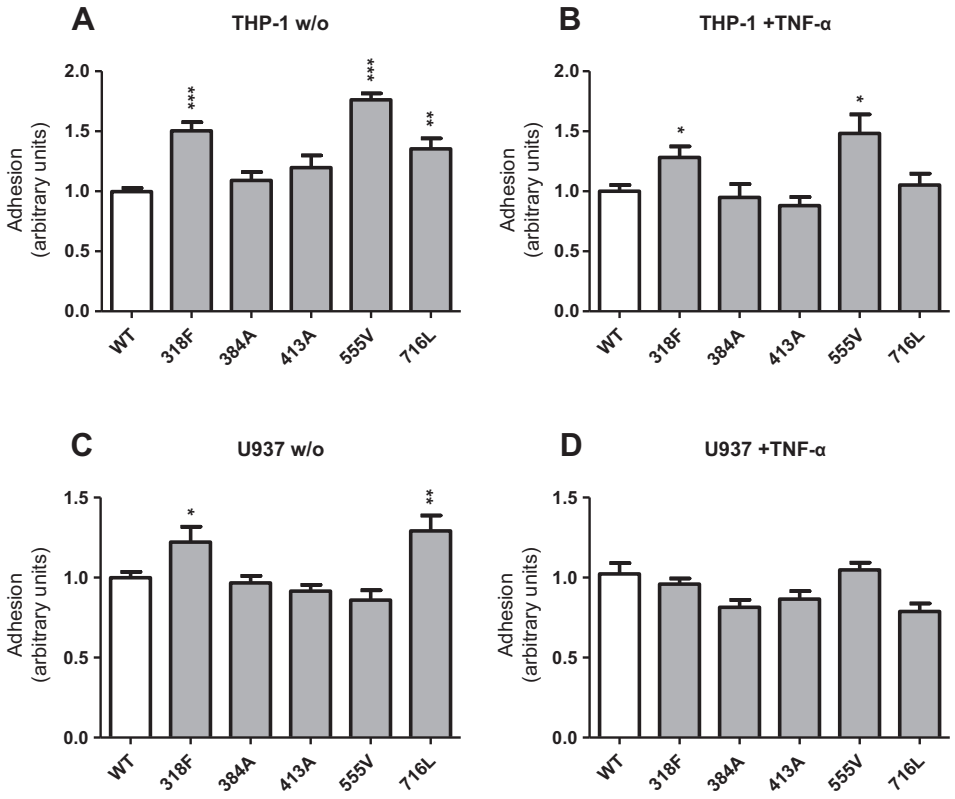

Fig. 5. Adhesion of monocytes to endothelial cells is significantly increased by VCAM-1 variants. (A) Cell adhesion of THP-1 cells under basic conditions (w/o) was significantly ($p < 0.01$) increased in the presence of 318F, 555V and 716L compared to wild type (wt). (B) Comparable results were obtained with TNF-α-treated endothelial cells ($p < 0.05$), except for variant 716. (C) Cell adhesion of U937 cells under basic conditions was significantly increased in the presence of 318F and 716L compared to wild type (wt). (D) No significant differences in U937 cell adhesion were observed for TNF-α-treated endothelial cells. Adhesion assays were performed 24 h after transfection of endothelial cells in triplicates and are shown as standard deviation of the mean. Adhesion assays under basic conditions were repeated at least 4 times; adhesion assays after TNF-α stimulation at least 2 times. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$.

second IDPSPL site is present in module 4 (domain IV). Although both modules interact with α4β1, module 1, based on the close proximity to the cell surface, facilitates robust α4β1-mediated adhesion of blood eosinophils. By contrast, reduced adhesion to VCAM-1 containing only modules 4–7 has been reported [20], underlining the functional difference of the two domains in cell-adhesion. Studies with leukaemic cell lines have indicated that recognition of module 1 and 4 by eosinophils is a complex process, which could not be explained by α4β1 expression level and activation state alone [21]. The relevance of domain IV in pathophysiological processes *in vivo* has been highlighted by VCAM-1 domain 4-deficient (D4D) mice, which are protected against atherosclerosis [8,9]. In the present analysis, two of the analysed VCAM-1 variants are located in domain IV, S318F and T384A. Overexpression of the VCAM-1 form 318F in endothelial cells significantly increased adhesion of both monocytic cell lines, THP-1 and U937, under basic conditions. The effect was also observed for THP-1 monocytes when endothelial cells were stimulated with TNF-α. S318F exchanges the polar amino acid serine for a more hydrophobic phenylalanine and is qualified to be potentially damaging by the *in silico* tool PolyPhen (http://genetics.bwh.harvard.edu/pph/). Notably, overexpression of VCAM-1 form 384A, which changes a polar amino acid to a non-polar aliphatic amino acid, did not affect cell adhesion in any of our experimental settings. Our results indicate that the observed effect on increased cell–cell adhesion is based on a structural change of the VCAM-1 protein due to insertion of 318F, whereas a change in total VCAM-1 protein due to this variant seems unlikely. Even if all analysed variants including 318F affected VCAM-1 mRNA level, an effect that has been reported for a broad range of variants in the transcribed region interfering with mRNA processing and turnover [22], this did not translate into detectable alterations of total VCAM-1 protein levels.

VCAM-1 variant I716L, located within the transmembrane domain, represents an amino acid exchange from non-polar to basic. We observed increased VCAM-1 protein levels of up to 50% in endothelial cells overexpressing 716L using a sensitive ELISA. Consistently, THP-1 and U937 adhesion to endothelial cells overexpressing 716L was significantly increased. It is conceivable that increased cell–cell adhesion caused by this variant is based on elevated VCAM-1 protein levels, which might originate from altered interaction with cellular membranes and modified protein turnovers. We also observed enhanced monocyte adhesion to endothelial cells overexpressing 555V, which is not located in any functional domain of the VCAM-1 protein. However, domain VI has been reported to include two N-glycosylation sites [23] and 555V might alter protein folding which interferes with posttranslational glycosylation processes. Interestingly, we have observed differences between the two monocytic cell lines THP-1 and U937 with respect to cell adhesion in presence of the three VCAM-1 forms 318F, 555v and 716L. While 318F and 716L altered adhesion of both cell lines, 555V affected only the interaction of THP-1 monocytes and endothelial cells. Differences in the adhesion of the two monocytic cell lines included in our study have been reported previously [24], pointing towards a potentially distinct mechanism involved in the adhesion of the two cell lines.

The here described observation suggests that VCAM-1 variants affect monocyte adhesion to endothelial cells *in vitro* due to distinct mechanisms depending on the inserted variant. The effects potentially include modification of total VCAM-1 protein level in

case of 716L and modification of VCAM-1 protein structure and posttranslational processes in case of 318F and 555V. Observed alterations in VCAM-1 mRNA levels may be based on altered folding energies but do not directly translate into altered cell adhesion.

5. Conclusion

In conclusion, VCAM-1 variants 318F, 555V and 716L significantly increased the adhesion of monocytes to endothelial cells *in vitro*. The observed effects rely on different molecular mechanisms and differ between monocytic cells. This observation may be of particular relevance for chronic inflammatory pathophysiologic conditions involving cell–cell adhesion such as atherosclerosis and other proinflammatory conditions. The impact of the analysed variants may point towards the distinct function of the different VCAM-1 domains in cell type-specific adhesion. The described effects could differ between endothelial cells of different origin and other cells and tissues, since we observed different effects in CV1 fibroblasts. Further elucidation of the precise mechanisms altered by 318F and 555V and their temporal dynamics needs to be addressed by more extensive approaches such as surface plasmon resonance and functional consequences should be further studied in appropriate animal models.

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Conflict of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2013.07.039.

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